Role and Regulation of STAT3 Phosphorylation at Ser727 in Melanocytes and Melanoma Cells

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The transcription factor signal transducer and activator of transcription 3 (STAT3) has two important phosphorylation sites, Tyr705 and Ser727, for its activation. Ser727 phosphorylation has been considered to be a secondary event after Tyr705 phosphorylation. In this study, the role and regulation of Ser727 phosphorylation in STAT3 in melanocytic cells were examined. STAT3 was phosphorylated on Ser727 in the absence of Tyr705 phosphorylation in melanocytes. 12-*O*-tetradecanoylphorbol-13-acetate-induced increase in cell survival activity and nuclear translocation of STAT3 was associated with Ser727 phosphorylation. Ser727 was constitutively phosphorylated in all melanoma cell lines examined irrespective of Tyr705 phosphorylation. The possible involvement of Ser727 phosphorylation in STAT3 in cell survival activity and nuclear translocation of STAT3 in melanocytes was demonstrated also in melanoma cells. The constitutive Ser727 phosphorylation in melanoma cells was partially mediated by the B-Raf–MEK–ERK1/2 pathway. Immunohistochemical studies on specimens of primary lesions of acral lentiginous melanoma revealed that Ser727 phosphorylation precedes Tyr705 phosphorylation in the early stages of melanoma progression. Our results indicate that Ser727 phosphorylation on STAT3 is not necessarily a secondary event after Tyr705 phosphorylation and suggest that it has a role in the regulation of cell survival activity and nuclear translocation of STAT3 in melanocytic cells.

Journal of Investigative Dermatology (2012) **132,** 1877–1885; doi:10.1038/jid.2012.45; published online 15 March 2012

INTRODUCTION

Malignant melanoma arises from the transformation and proliferation of melanocytes that are present in the basal layer of the skin epidermis and are responsible for the production of the skin pigment melanin. Although there have been significant basic scientific advances in understanding of the tumor biology of melanoma (Smalley and Herlyn, 2005; Hocker *et al.*, 2008; Lopez-Bergami *et al.*, 2008; Russo *et al.*, 2009), the exact mechanism for melanomagenesis has not been fully elucidated.

The transcription factor signal transducer and activator of transcription 3 (STAT3) is activated in response to various growth factors, hormones, and cytokines, and has an important role in their signaling (Darnell, 1997). When these ligands bind to the specific transmembrane STAT3 receptor, STAT3 becomes activated by JAK-mediated tyrosine phosphorylation of a critical tyrosine residue (Tyr705) and dimerizes through reciprocal Src homology 2–phosphotyrosine interaction. The dimeric STAT3 translocates to the nucleus, where it binds to consensus STAT3 binding sequences within the promoter region of target genes and thereby activates their transcription (Darnell, 1997). It is generally thought that tyrosine phosphorylation regulates the dimerization of STATs as an essential prerequisite for the establishment of a classical JAK–STAT3 signaling pathway and Tyr705 phosphorylation has been regarded as a characteristic of STAT3 activation (Bowman *et al.*, 2000; Decker and Kovarik, 2000; Levy and Lee, 2000; Aggarwal *et al.*, 2009). However, the STAT3 molecule contains a second phosphorylation site, Ser727, within its C-terminus. Although it has been suggested that Ser727 phosphorylation is a secondary event after Tyr705 phosphorylation required for the maximal transcriptional activity of STAT3 (Wen *et al.*, 1995), it is now becoming clear that regulation of STAT3 function by Ser727 phosphorylation is more complex than originally expected (Bowman *et al.*, 2000; Decker and Kovarik, 2000; Aggarwal *et al.*, 2009).

In normal cells, the duration of STAT3 activation is temporary, usually lasting from a few minutes to several hours (Bromberg and Chen, 2001). In contrast, constitutive activation of STAT3, as demonstrated by detection of Tyr705 phosphorylation, has been observed in many kinds of tumors including melanomas (Niu *et al.*, 2002; Messina *et al.*, 2008; Oka *et al.*, 2009). We have recently demonstrated that constitutive phosphorylation of Tyr705 has an important role in melanoma growth and this activation is

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Abbreviations: ALM, acral lentiginous melanoma; ERK, extracellular signal–regulated kinase; MEK, extracellular signal–regulated kinase kinase; pS-STAT3, STAT3 phosphorylated on Ser727; pY-STAT3, STAT3 phosphorylated on Tyr705; siRNA, small interfering RNA; STAT3, signal transducer and activator of transcription 3; TPA, 12-O-tetradecanoylphorbol-13-acetate

Received 8 August 2011; revised 16 January 2012; accepted 24 January 2012; published online 15 March 2012

negatively regulated by protein-kinase-C–activated tyrosine phosphatase(s) (Oka *et al.*, 2009). In the present study, we examined the role and regulation of Ser727 phosphorylation in STAT3 in melanocytic cells. We showed that STAT3 is constitutively phosphorylated on Ser727 irrespective of Tyr705 phosphorylation in normal melanocytes and melanoma cells and that the Ser727 phosphorylation is associated with cell survival activity and nuclear translocation of STAT3 in these cells. We also showed that the constitutive Ser727 phosphorylation is in part regulated by B-Raf-extracellular–regulated kinase (ERK) kinase (MEK) in melanoma cells. In addition, we demonstrated that Ser727 is frequently phosphorylated in the absence of Tyr705 phosphorylation in melanoma cells *in vivo*, particularly in *in situ* lesions of acral lentiginous melanoma (ALM) tissues.

RESULTS

Phosphorylation state of Ser727 in STAT3 in human melanocytes and melanoma cells

To determine the phosphorylation state of Ser727 in STAT3 in melanocytic cells, the expression levels of STAT3 phosphorylated on Ser727 (pS-STAT3) as well as on STAT3 phosphorylated on Tyr705 (pY-STAT3) in melanocytes and seven human melanoma cell lines were examined by western blotting (Figure 1). In cultured quiescent melanocytes, low levels of pS-STAT3 but not pY-STAT3 were detected. pS-STAT3 was expressed in all the seven melanoma cell lines, whereas pY-STAT3 level was not detected in two melanoma cell lines, WM35 and WM39 cells.

Association of Ser727 phosphorylation with cell survival activity and nuclear translocation of STAT3 in melanocytes

We have previously shown that the tumor-promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) has an anti-apoptotic effect on growth factor deprivation-induced apoptosis in melanocytes (Oka *et al.*, 2004). To examine the possible involvement of Ser727 phosphorylation in the protection against growth factor deprivation-induced apoptosis in melanocytes, the effects of TPA on pS-STAT3 expression and on growth factor deprivation-induced apoptosis in melanocytes was examined (Figure 2a). In quiescent melanocytes, ERK1/2 was phosphorylated slightly as described previously (Oka *et al.*, 2007). Upon treatment with TPA, the expression of pS-STAT3 was enhanced in a time-dependent manner concomitant with an increase in ERK1/2 activity within 60 minutes of treatment. Thereafter, both the pS-STAT3 expression level and ERK1/2 activity decreased to basal levels by 24 hours. The increase in the expression of pS-STAT3 was associated with the increase in cell survival activity of the melanocytes (Figure 2b).

To determine the effect of TPA on the cellular localization of pS-STAT3 in melanocytes, immunofluorescent analysis was carried out (Figure 2c and d). In quiescent cells, very low levels of pS-STAT3 were detected in the nucleus (Figure 2c, pS-STAT3, 0 hours). Total STAT3 was localized mainly in the cytoplasm (Figure 2d, t-STAT3, 0 hours). At 1 hour after TPA treatment, nuclear pS-STAT3 as well as total nuclear STAT3 increased (Figure 2c, pS-STAT3, 1 hour and Figure 2d, t-STAT3, 1 hour). TPA-induced increase in nuclear pS-STAT3 and total nuclear STAT3 was confirmed also by western blotting (Supplementary Figure S1 online). In addition, western blotting revealed that TPA increases cytoplasmic pS-STAT3. Data from immunofluorescent and western blot analyses suggest that TPA induces Ser727 phosphorylation of cytoplasmic STAT3 and that a part of the increased cytoplasmic pS-STAT3 actively translocates into the nucleus.

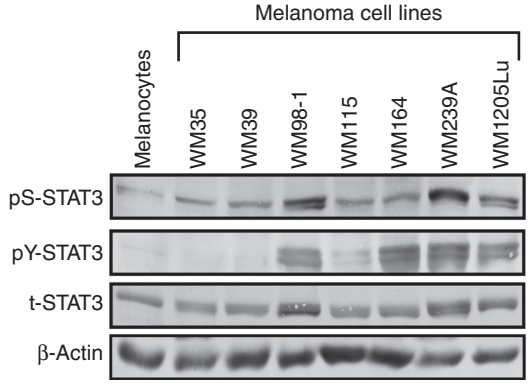

Figure 1. Ser727 is constitutively phosphorylated in signal transducer and activator of transcription 3 (STAT3) in human melanocytes and melanoma cells. Whole-cell extracts from quiescent melanocytes and seven human melanoma cell lines, WM35, WM39, WM98-1, WM115, WM164, WM239-A, and WM1205Lu, were prepared, and the phosphorylation levels of STAT3 phosphorylated on Ser727 (pS-STAT3) as well as of STAT3 phosphorylated on Tyr705 (pY-STAT3) were determined by western blotting using anti-pS-STAT3 and anti-pY-STAT3 antibodies, respectively. The expression levels of total STAT3 (t-STAT3) and β-actin were also examined. The results shown are representative of three independent experiments.

Regulation of the constitutive phosphorylation of Ser727 in STAT3 in melanoma cells

We next focused on Ser727 phosphorylation in melanoma cells. It has been reported that several kinases, such as ERK1/2 (Chung *et al.*, 1997), p38 mitogen-activated protein kinase (Gollob *et al.*, 1999), c-Jun N-terminal kinase (Lim and Cao, 1999), protein kinase C (Jain *et al.*, 1999; Aziz *et al.*, 2007), mammalian target of rapamycin (Yokogami *et al.*, 2000), and cyclin-dependent kinase 5 (Fu *et al.*, 2004), mediate Ser727 phosphorylation in STAT3. To examine whether these kinases are involved in the constitutive phosphorylation of Ser727 in STAT3 in melanoma cells, the effects of inhibitors of these kinases on the phosphorylation state of Ser727 in four melanoma cell lines, WM35, WM39, WM98-1, and WM1205Lu, were investigated. U0126, which inhibits ERK1/2 through MEK inhibition, reduced the phosphorylation of pS-STAT3 by approximately 30–50% in three cell lines, WM39, WM98-1, and WM1205Lu, concomitant with inhibition of ERK1/2 activity (Figure 3a). Conversely, inhibitors of p38 mitogen-activated protein kinase (SB203580), c-Jun N-terminal kinase (SP600125), protein kinase C (GF109203X), mammalian target of rapamycin kinase (rapamycin), and cyclin-dependent kinase 5 (roscovitine) did not affect the phosphorylation level of pS-STAT3 in these four cell lines (Supplementary Figure S2 online). U0126 treatment

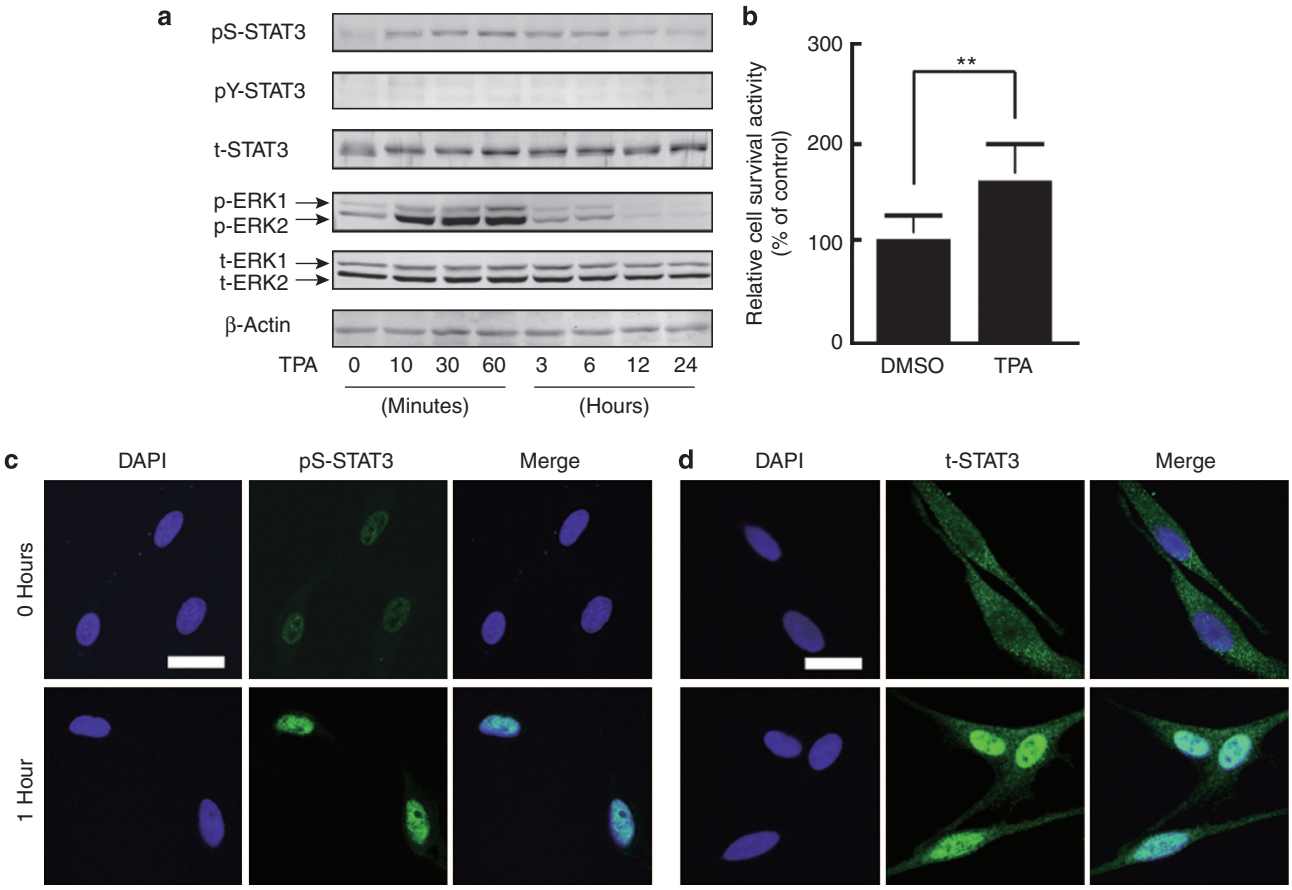

Figure 2. 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced cell survival activity and nuclear translocation of signal transducer and activator of transcription 3 (STAT3) are associated with Ser727 phosphorylation in melanocytes. (**a**) Quiescent melanocytes were treated with 100 nM TPA. The expression levels of STAT3 phosphorylated on Ser727 (pS-STAT3), STAT3 phosphorylated on Tyr705 (pY-STAT3), total STAT3 (t-STAT3), phosphorylated extracellular signal–regulated kinase (ERK)1/2 (p-ERK1 and p-ERK2), total ERK1/2 (t-ERK1 and t-ERK2), and β-actin were determined at the indicated time as in Figure 1. (**b**) Quiescent melanocytes were treated with either 0.1% DMSO (control) or 100 nM TPA for 24 hours, and cell survival activity was assessed. Data are expressed as percentage of control (DMSO) and are shown as mean ± SD, ($n = 3$; $^{**}P < 0.01$). (**c**, **d**) Quiescent melanocytes were treated with 100 nM TPA for 1 hour. The localization of (**c**) pS-STAT3 as well as (**d**) total STAT3 before (0 hours) and after (1 hour) the treatment was examined by immunofluorescent analysis. Bar = 20 μm.

increased pY-STAT3 expression in WM98-1 and WM1205Lu cells (Figure 3a). The mechanism of U0126-induced upregulation of pY-STAT3 expression in these cells is currently unknown.

It has been shown that an activating mutation in B-Raf (V600E) frequently occurs in melanoma cells (Davies *et al.*, 2002), leading to activation of the MEK–ERK1/2 pathway (Heath *et al.*, 2011). To examine whether an activating mutation of B-Raf is involved in the MEK–ERK1/2-induced Ser727 phosphorylation in STAT3 in melanoma cells, the effect of the inhibitor of active B-Raf (V600E) kinase, PLX4720 (Tsai *et al.*, 2008), on the phosphorylation state of Ser727 in the four melanoma cell lines WM35, WM39, WM98-1, and WM1205Lu was examined (Figure 3b). Of the four melanoma cell lines, WM35, WM39, and WM1205Lu cells harbor the B-Raf V600E mutation (Smalley *et al.*, 2008), while the B-Raf V600E mutation has not been examined in WM98-1 cells. As shown in Figure 3b, inhibition of active B-Raf kinase reduced pS-STAT3 expression in all the four melanoma cell lines, indicating that Ser727 phosphorylation in STAT3 is partly mediated by the B-Raf–MEK–ERK1/2 pathway in melanoma cells.

Role of Ser727 phosphorylation in nuclear translocation of STAT3 in melanoma cells

To examine the relationship between the cellular localization of STAT3 and Ser727 phosphorylation in melanoma cells, the effect of U0126 on pS-STAT3 as well as total STAT3 was investigated by immunofluorescent analysis using WM39 cells containing pS-STAT3 but not pY-STAT3. pS-STAT3 was exclusively localized in the nucleus of the cells (Figure 3c, pS-STAT3, 0 hours). Total STAT3 was detected strongly in the nucleus and weakly in the cytoplasm (Figure 3d, t-STAT3, 0 hours). The treatment of cells with U0126 resulted in a decrease in nuclear pS-STAT3 as well as in total nuclear STAT3 concomitant with an increase in total cytoplasmic STAT3 (Figure 3c and d, pS-STAT3 and t-STAT3, 2 hours), suggesting that dephosphorylated nuclear STAT3 translocates into the cytoplasm.

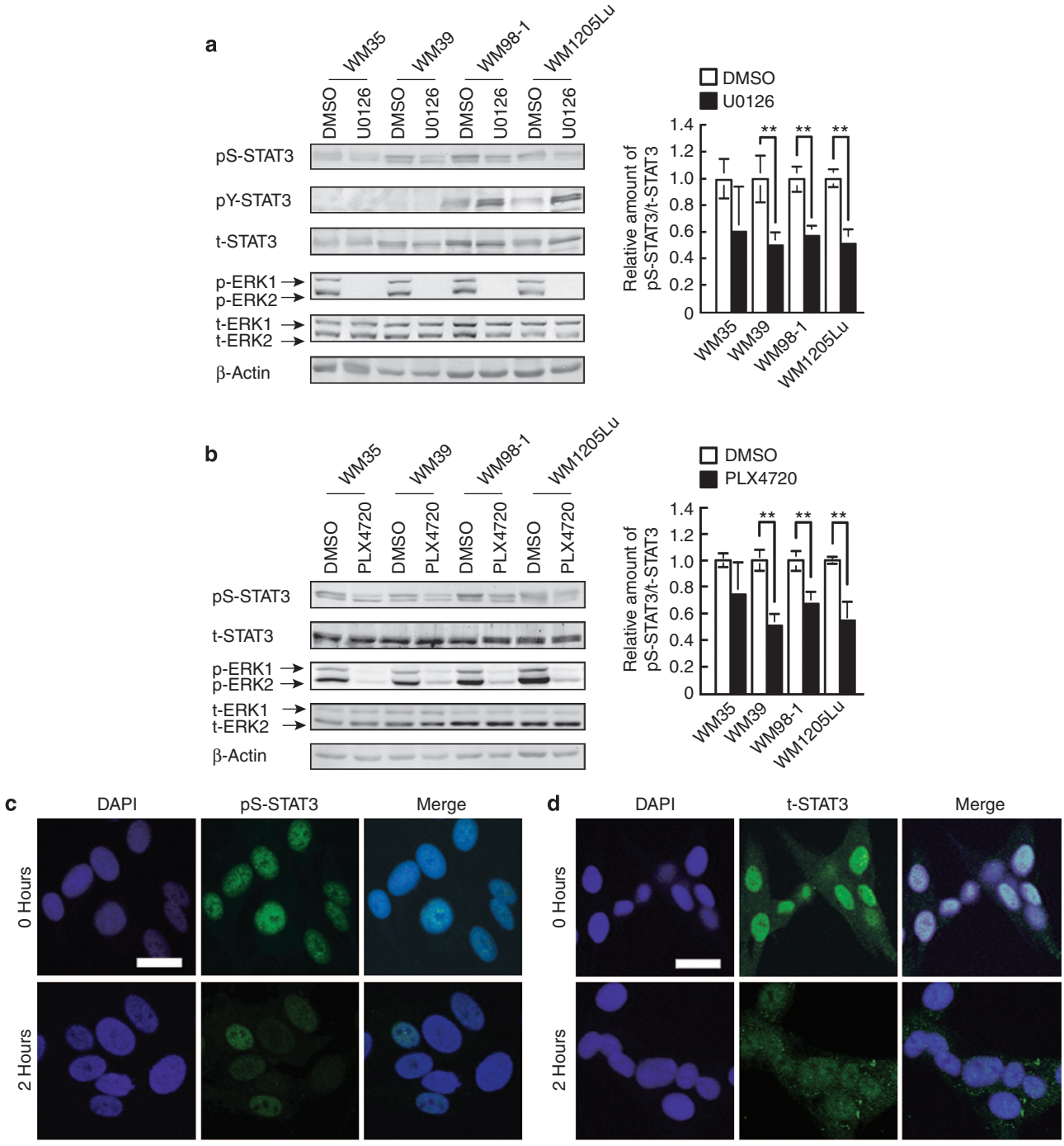

Figure 3. Regulation and role of Ser727 phosphorylation in melanoma cells. Melanoma cells were treated with (**a**) either 25 μM U0126 for 2 hours or (**b**) 1 μM PLX4720 for 3 hours. (**a** and **b**, left) The expression levels of signal transducer and activator of transcription 3 (STAT3) phosphorylated on Ser727 (pS-STAT3), STAT3 phosphorylated on Tyr705 (pY-STAT3), total STAT3 (t-STAT3), extracellular signal–regulated kinase (ERK)1/2, total ERK1/2, and β-actin in treated melanoma cells were determined by western blotting as in Figure 1. (**a** and **b**, right) The relative amount of pS-STAT3/t-STAT3 in treated cells was calculated. The ratio of pS-STAT3/t-STAT3 in DMSO-treated (control) cells is represented as 1. Data are shown as mean ± SD ($n = 3$; $^{**}P < 0.01$). (**c**, **d**) WM39 cells were treated with 25 μM U0126 for 2 hours and the localization of (**c**) pS-STAT3 as well as (**d**) t-STAT3 before (0 hours) and after (2 hours) the treatment were examined by immunofluorescent analysis. Bar = 20 μm.

It has been shown that activation (Tyr705 phosphorylation) of STAT3 in some melanoma cell lines relies on JAK (Kreis *et al.*, 2007). To exclude the possibility of the involvement of Tyr705 phosphorylation in the nuclear translocation of STAT3, the effects of a JAK inhibitor, JAK inhibitor I, on the cellular localization of STAT3 in WM39 cells were examined. Cellular localization of pS-STAT3 as well as of total STAT3 was not affected either at 1 hour (data

not shown) or at 3 hours after JAK inhibitor I treatment in WM39 cells (Supplementary Figure S3 online), indicating that the JAK–pY-STAT3 pathway is not involved in the nuclear translocation of STAT3.

Association of Ser727 phosphorylation with cell survival activity in melanoma cells

The effect of the small interfering RNAs (siRNAs) of STAT3 on cell survival activity was examined in WM39 cells. Knockdown of STAT3 by siRNAs in WM39 cells significantly reduced the expression levels of total STAT3 as well as of pS-STAT3 concomitant with a decrease in cell survival activity (Figure 4a and b). As the majority of STAT3 is present as pS-STAT3 in WM39 cells, these results indicate that the expression level of pS-STAT3 is associated with the cell survival activity of melanoma cells. The association between the expression level of pS-STAT3 and the cell survival activity of melanoma cells was also shown in an experiment in which the effects of U0126 on cell survival activity of melanoma cells were examined (Figure 4c). U0126 treatment significantly reduced the cell survival activity of all the four melanoma cell lines examined.

Detection of DNA-binding activity of pS-STAT3 in melanoma cells and melanocytes

To determine whether pS-STAT3 has DNA-binding activity in the absence of Tyr705 phosphorylation, the effects of U0126 or TPA treatment on the DNA-binding activity of STAT3 in WM39 melanoma cells and melanocytes, respectively, were investigated. Significant changes in the DNA-binding activity of STAT3 between non-treated and treated cells were not detected in either WM39 cells or melanocytes (Supplementary Figure S4 online).

Expression levels of pS-STAT3 and pY-STAT3 in melanoma tissues

Finally, the expression levels of pS-STAT3 and pY-STAT3 were examined in 15 sections of primary lesions of ALM, which is the most common clinical type in Japan and has clear *in situ* lesions in the early stages of progression (Seiji *et al.*, 1983) (Figure 5 and Table 1). The 15 sections were divided into two groups by histological progression, i.e., *in situ* only group (nine cases) and *in situ* plus dermal invasion group (six cases). In the nine cases of the *in situ* only group, pS-STAT3 but not pY-STAT3 was detected mainly in the nucleus in some but not all of the melanoma cells in five cases. Neither pS-STAT3 nor pY-STAT3 was detected in the remaining four cases of *in situ* only group. In the six cases of the *in situ* plus dermal invasion group, pS-STAT3 was detected in some but not all melanoma cells, mainly in the nucleus, in both *in situ* and dermal invasive lesions in all cases, while pY-STAT3 was detected in some cells, mainly in the nucleus, in only dermal invasive lesions in four cases.

DISCUSSION

In the case of polypeptide ligand-induced STAT3 activation in cells, STAT3 translocates to the nucleus after being phosphorylated at Tyr705, followed by phosphorylation at Ser727. Thus, it has been assumed that Ser727 phosphorylation is a secondary event after Tyr705 phosphorylation, and Tyr705 phosphorylation has been regarded as a characteristic of STAT3 activation. However, we have found in this study that Ser727 is constitutively phosphorylated in melanocytes and melanoma cells irrespective of Tyr705 phosphorylation. These results suggest that the regulation mechanism of Ser727 phosphorylation in melanocytic cells is different from that of polypeptide ligand-induced Ser727 phosphorylation. We have shown that Ser727 is constitutively phosphorylated in the absence of Tyr705 phosphorylation in melanocytes and that TPA increases the phosphorylation level of Ser727 without inducing Tyr705 phosphorylation. Thus, it is evident that TPA-induced Ser727 phosphorylation in melanocytes is not a secondary event after Tyr705 phosphorylation, although the detailed mechanism of TPA-induced Ser727 phosphorylation remains unclear. On the other hand, we have shown that constitutive Ser727 phosphorylation in melanoma cells is partially regulated by the B-Raf–MEK–ERK1/2 pathway. As the Pro-Met-Ser727-Pro sequence at the C-terminus of STAT3 (Wen *et al.*, 1995) has been identified as a phosphorylation site of ERKs, Pro-*X*-Ser-Pro (Gonzalez *et al.*, 1991), our results

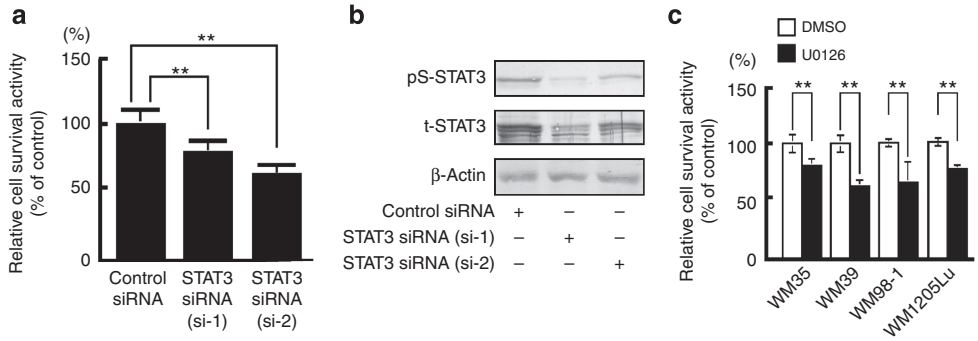

Figure 4. Expression level of signal transducer and activator of transcription 3 (STAT3) phosphorylated on Ser727 (pS-STAT3) is associated with cell survival activity in melanoma cells. (**a**) WM39 cells were transfected with control small interfering RNA (siRNA; 40 nM) or STAT3 siRNAs, si-1 and si-2 (40 nM). On the following day, cell survival activity was assessed. Data are shown as mean ± SD ($n=3$; $^{**}P<0.01$). (**b**) Twenty-four hours after siRNA transfection, the expression levels of pS-STAT3, total STAT3 (t-STAT3), and β-actin in WM39 cells were analyzed as in Figure 1. (**c**) Melanoma cells were treated with either 0.1% DMSO (control, white bars) or 25 μM U0126 (black bars) for 3 hours, and cell survival activity was assessed. Data are shown as mean ± SD ($n=3$; $^{**}P<0.01$).

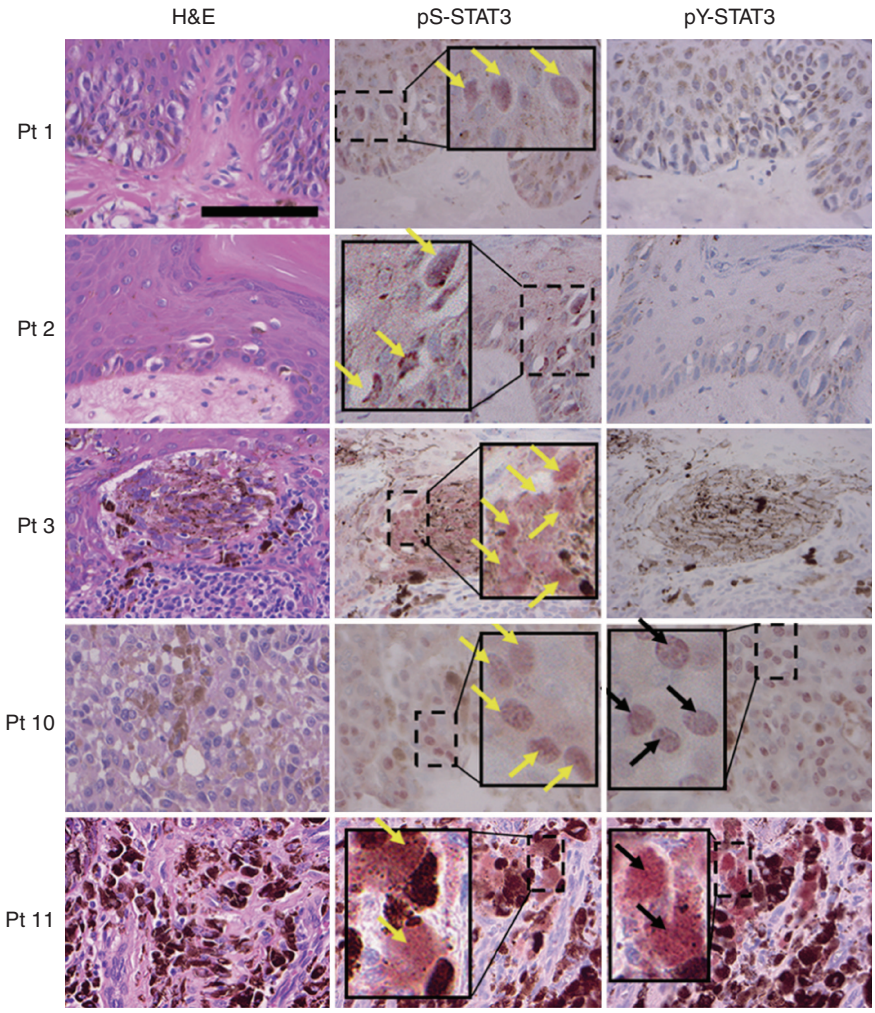

Figure 5. Expression of signal transducer and activator of transcription 3 (STAT3) phosphorylated on Ser727 (pS-STAT3) and STAT3 phosphorylated on Tyr705 (pY-STAT3) in primary acral lentiginous melanoma (ALM) tissues. Sections from ALM patients were immunostained with antibodies against pS-STAT3 and pY-STAT3. The yellow and black arrows indicate positive immunolocalization of pS-STAT3 and pY-STAT3, respectively. Representative results of three patients (Pt 1, Pt 2, and Pt 3) with ALM *in situ* and two patients (Pt 10 and Pt 11) with ALM containing dermal invasive lesions as well as *in situ* lesions are shown (bar = 100 μm). Patient numbers correspond to those in Table 1. Insets show magnified images of pS-STAT3- or pY-STAT3-positive cells in dotted squares, respectively. Atypical melanocytes stain for pS-STAT3 but not pY-STAT3 (Pt 1, Pt 2, and Pt 3). Melanoma cells in the dermis stain for both pS-STAT3 and pY-STAT3 (Pt 10 and Pt 11). H&E, hematoxylin and eosin.

suggest that activating mutations in B-Raf contribute to Ser727 phosphorylation in STAT3 in melanoma cells through the MEK–ERK1/2 pathway.

The constitutive phosphorylation of Ser727 in melanocytes and melanoma cells suggests that Ser727 phosphorylation has a role in these cells. The close association of the pS-STAT3 expression level with the nuclear translocation of STAT3 and cell survival activity suggests that Ser727 phosphorylation is involved in the regulation of cell survival activity and nuclear translocation of STAT3 in melanocytic cells. We have previously shown that Tyr705 phosphorylation is important for melanoma growth, suggesting that it is likely that both pS-STAT3 and pY-STAT3 have a role either synergistically or independently in survival or growth of melanoma cells. However, our present study suggests that pS-STAT3 may have a role in cell survival in melanocytes in the absence of pY-STAT3. Collectively, we consider that the function of pS-STAT3 in the enhancement of cell survival activity in melanocytes is active during melanomagenesis, while the function of pY-STAT3 in survival and growth appears in melanoma cells, and works in addition to that of pS-STAT3. Our immunofluorescent analysis data indicate the presence of small amounts of unphosphorylated STAT3 in the nucleus in melanocytes and melanoma cells. The role of the unphosphorylated STAT3 in gene regulation in these cells remains to be clarified.

As STAT3 is a transcription factor and pS-STAT3 is localized mainly in the nucleus, it was assumed that the stimulatory effect of pS-STAT3 on cell survival activity in melanocytic cells is mediated by its transcriptional activity after DNA binding. It has been shown that Ser727 phosphorylation regulates the transcriptional activity of STAT3

Table 1. Summary of expression of pS-STAT3 and pY-STAT3 in ALM tissues

		% Of pS-STAT3-positive melanoma cells[1]		% Of pY-STAT3-positive melanoma cells[1]	
Patient number	Histological progression	Epidermis	Dermis	Epidermis	Dermis
1	*In situ* only	64 ± 46	0	0	0
2	*In situ* only	52 ± 30	0	0	0
3	*In situ* only	74 ± 23	0	0	0
4	*In situ* only	32 ± 8.4	0	0	0
5	*In situ* only	54 ± 21	0	0	0
6	*In situ* only	0	0	0	0
7	*In situ* only	0	0	0	0
8	*In situ* only	0	0	0	0
9	*In situ* only	0	0	0	0
10	*In situ* + dermal invasion	64 ± 25	16 ± 11	0	54 ± 21
11	*In situ* + dermal invasion	60 ± 35	20 ± 10	0	38 ± 13
12	*In situ* + dermal invasion	72 ± 29	36 ± 12	0	66 ± 30
13	*In situ* + dermal invasion	66 ± 26	42 ± 17	0	72 ± 22
14	*In situ* + dermal invasion	48 ± 23	22 ± 16	0	0
15	*In situ* + dermal invasion	20 ± 14	28 ± 13	0	0

Abbreviations: ALM, acral lentiginous melanoma; pS-STAT3, signal transducer and activator of transcription 3 (STAT3) phosphorylated on Ser727; pY-STAT3, STAT3 phosphorylated on Tyr705.
[1]The percentage of pS-STAT3- or pY-STAT3-positive cells in the epidermis or dermis was determined by averaging the pS-STAT3- or pY-STAT3-positive cell counts in five randomly chosen regions of the skin section. Data are expressed as the mean ± SD.

positively (Schuringa *et al.*, 2001; O'Rourke and Shepherd, 2002; Aziz *et al.*, 2010) or negatively (Jain *et al.*, 1999) in the presence of Tyr705 phosphorylation. However, it has not been determined whether pS-STAT3 has DNA-binding activity in the absence of Tyr705 phosphorylation. We attempted to detect pS-STAT3-dependent but pY-STAT3-independent DNA-binding activity by comparing the activity between U0126-treated and U0126-untreated WM39 cells and between TPA-treated and TPA-untreated melanocytes using the TransAM STAT3 transcription factor assay kit, but no significant difference in DNA-binding activity was detected in either case (Supplementary Figure S4 online). This does not exclude the possibility that pS-STAT3 binds to DNA, leading to transcriptional regulation of genes. The principle of the kit is that it contains a 96-well plate with immobilized oligonucleotides encoding a pY-STAT3 consensus site (5′-TTCCCGGAA-3′) (Zhong *et al.*, 1994; Darnell, 1997) and the active form of STAT3 contained in the cell extract should specifically bind to these oligonucleotides. Thus, if pS-STAT3 binds to a different site(s) from the pY-STAT3 consensus site, the DNA-binding activity of pS-STAT3 cannot be detected by the kit. From the data obtained in the present study, including Supplementary Figure S4 online, it is considered that pS-STAT3 has its own target genes that have binding site(s) for pS-STAT3 but not for pY-STAT3.

In accordance with *in vitro* data, immunohistochemical studies on specimens of primary lesions of ALM revealed that pS-STAT3 is frequently expressed in the absence of Tyr705 phosphorylation in melanoma cells in *in situ* lesions of ALM, and that pY-STAT3 as well as pS-STAT3 is expressed in dermal invasive lesions of ALM. These data raise two possibilities regarding the roles of Ser727 and Tyr705 phosphorylation. One is that each of Ser727 phosphorylation and Tyr705 phosphorylation has its own role and affects different functions of STAT3. In this case, it is interesting to consider whether Ser727 phosphorylation has a role in the acquisition of autonomous growth in the early stages of ALM progression, especially in *in situ* lesions, and whether Tyr705 phosphorylation acts as a molecular switch to the dermal invasion by melanoma cells. The other possibility is that Ser727 phosphorylation and Tyr705 phosphorylation act synergistically on STAT3 functions.

To summarize, our results indicate that Ser727 phosphorylation on STAT3 is not necessarily a secondary event after Tyr705 phosphorylation and that Ser727 phosphorylation has its own role and regulation mechanism in melanocytic cells. Elucidation of the detailed regulation mechanism of Ser727 phosphorylation and the role of pS-STAT3 may lead to a better understanding of melanomagenesis.

MATERIALS AND METHODS

Antibodies and reagents

Antibodies against STAT3 and pS-STAT3 were from Santa Cruz Biotechnology (Santa Cruz, CA). An antibody against pY-STAT3 was obtained from Cell Signaling Technology (Beverly, MA). Antibodies against ERK1/2 and phospho-ERK1/2 were purchased from Promega (Madison, WI). An antibody against β-actin was from Epitomics.

(Burlingame, CA). TPA was purchased from Sigma (St Louis, MO). DMSO was obtained from Nacalai Tesque (Kyoto, Japan). The MEK inhibitor U0126 was from Sigma. The inhibitors for p38, mitogen-activated protein kinase (SB203580), c-Jun N-terminal kinase (SP600125), protein kinase C (GF109203X), mammalian target of rapamycin kinase (rapamycin), cyclin-dependent kinase 5 (roscovitine), active B-Raf (V600E) kinase (PLX4720), and JAK (JAK inhibitor I), were purchased from Calbiochem (San Diego, CA).

Cells and cell culture

Seven human melanoma cell lines from primary (WM35, WM39, WM98-1, and WM115) and metastatic (WM164, WM239A, and WM1205Lu) lesions were kindly provided by Dr Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). The melanoma cell lines were cultured in EMEM containing 5% fetal calf serum as described previously (Oka *et al.*, 2009). Normal human melanocytes were obtained and maintained as described previously (Oka *et al.*, 2007). Where indicated, melanocytes were rendered quiescent by culturing the cells in the absence of growth factors for 24 hours.

Western blotting

Western blotting was carried out as described previously (Oka *et al.*, 2007).

STAT3 siRNA

STAT3 siRNA was performed as described previously (Oka *et al.*, 2009) with double-stranded siRNAs against STAT3 (si-1: 5′-AAC UUCAGACCCGUCAACAAAdTdT-3′; 3′-dTdTGAAGUCUGGGCA GUUGUUU-5′ and si-2: AAC AUC UGC CUA GAU CGG CUA dTdT-3′ 3′-dTdT GUA GAC GGA UCU AGC CGA U-5′).

Measurement of the DNA-binding activity of STAT3

The DNA-binding capacity of STAT3 was determined using the TransAM STAT3 transcription factor assay kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions as described previously (Oka *et al.*, 2009).

Assay for cell survival activity

Cell survival activity was determined using a modified 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazoliumbromide assay employing the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Immunofluorescence

Cells were seeded on Laboratory-Teck chamber slides (Nalge Nunc International, Naperville, IL) and incubated at 37 °C with 5% CO_2. Forty-eight hours later, they were fixed for 30 minutes in 4% paraformaldehyde and permeabilized for 10 minutes with ice-cold methanol at −20 °C. The fixed cells were incubated for 1 hour in blocking solution (5% BSA with 0.1% triton X-100 in phosphate-buffered saline). Immunostaining was performed by incubating the slides with rabbit polyclonal anti-pS-STAT3 (1:400) or anti-STAT3 (1:400) at 4 °C overnight, followed by Alexa fluor 488 donkey anti-rabbit (1:100) antibody for 1 hour at room temperature. Then, stained cells were mounted by DAPI Fluoromount G, a mounting agent including DAPI (Southern Biotech, Birmingham, AL). Images were captured using a Carl Zeiss LSM510 confocal microscope (Carl Zeiss Microimage, Thornwood, NY).

Immunohistochemistry

Human melanoma tissues were obtained from patients undergoing surgery for melanoma after their informed consent. Fifteen specimens of ALM were pathologically divided into ALM *in situ* (nine cases) and ALM with dermal invasive lesions as well as *in situ* lesions (six cases). Paraffin-embedded melanoma tissues were deparaffinized in xylene, rehydrated through graded concentrations of ethanol and washed with phosphate-buffered saline. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 13 minutes. For antigen retrieval, sections were heated in 10 mM sodium citrate buffer (pH 6.0), followed by blocking with Non-Specific Staining Blocking reagent (DakoCytomation, Carpinteria, CA). The slides were incubated with rabbit polyclonal anti-pS-STAT3 (1:200) or anti-pY-STAT3 (1:200) with normal rabbit IgG overnight at 4 °C. The slides were further incubated with horseradish peroxidase-labeled anti-rabbit polymer EnVision + System (DakoCytomation) at room temperature for 25 minutes. Immune complexes were visualized with Simple Stain AEC solution (Nichirei, Tokyo, Japan), conjugated with hematoxylin. Positive controls (using tissue samples from human psoriasis vulgaris) and negative controls (using non-specific rabbit antibody instead of the individual primary antibody) for pS-STAT3 and pY-STAT3 were stained using the same procedures (Supplementary Figure S5 online).

Statistics

Differences between results were assessed for significance using the Student's *t*-test. $P<0.05$ was considered to be statistically significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This study was supported by the Global Center of Excellence Program ''Global Center for Education and Research in Integrative Membrane Biology'' (to MS) and research grants (19591305 and 23591645 to MO) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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